AN UNEXPECTED ACTIVATION AND THIOESTER BINDING OF D- AND L-PHENYLALANINE BY THE HEAVY ENZYME OF GRAMICIDIN S SYNTHETASE

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1. Introduction

Gramicidin S synthetase consists of the light (100 000 dalton) and the heavy (280 000 dalton) enzyme. The light enzyme activates Phe and initiates synthesis by transferring the thioester-bound D-phenylalanyl group from the light to the heavy enzyme which activates L-Pro, L-Val, L-Orn and L-Leu and catalyzes the remaining (about 19) of the reactions required for synthesis of the cyclic decapeptide [1]. Recently, using heavy enzyme [2] not contaminated by the light enzyme, we have unexpectedly found that it activates and thioesterbinds L- and D-Phe. In the past the ATP-PP_i exchange reaction in the presence of Phe has been used as a test for the presence of light enzyme in the heavy. The present results show that this is not necessarily correct.

The thiol site involved in the binding of L- and D-Phe to the heavy enzyme in the absence of light enzyme does not participate in gramicidin S synthesis since the D-[^{14}C]Phe thioesterbound to the heavy enzyme after incubation with labelled phenylalanine could not be incorporated into gramicidin S. It is therefore not the site which has been suggested to be involved when the D-phenylalanyl group is transferred from the light to the heavy enzyme [3].

2. Methods

2.1. *Isolation of light and heavy enzyme of gramicidin S synthetase*

The method in [2] was used. The light enzyme was about 80% pure and the heavy enzyme gave only one band on polyacrylamide gel electrophoresis at pH 7.0, pH 8–9 and also in the presence of SDS–mercaptoethanol [2].

2.2. *ATP–$^{32}PP_i$ exchange reaction*

The ATP–$^{32}PP_i$ exchange reaction was carried out as in [2].

2.3. *Estimation of gramicidin S synthesis*

Gramicidin S synthesis was estimated by the Millipore filter assay in [4] using optimum conditions [2].

2.4. *Thioester binding of labelled amino acids to the enzymes*

The incubation mixture (0.2 ml) contained 10 mM potassium phosphate (pH 7.2), 5 mM ATP, 25 mM $MgCl_2$, 5 mM dithiothreitol, 6.25 μM EDTA and 0.01 mM amino [^{14}C]acid (spec. act. 100 Ci/mol) and enzyme. The reaction was stopped by adding 0.5 ml cold 20% trichloroacetic acid solution containing 0.1 M unlabelled amino acid and the mixture filtered on Millipore filter. The filter was washed with 25 ml 6% trichloroacetic acid and dried at 100°C for 10 min and put into vials containing 5 ml 0.4% PPO in toluene and counted.

2.5. *Liberation of thioester-bound amino [^{14}C]acid*

Performic acid oxidation of protein and identification of liberated material by two-dimensional thin-layer chromatography and radioautography were carried out as in [5].

Liberation of bound material by alkaline treatment of protein was carried out in 0.2 N NaOH at 37°C for 20 min.

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2.6. *Possible synthesis of gramicidin S after charging heavy enzyme with D-[^{14}C]Phe*

Heavy enzyme (about 400 μg) was charged with D-[^{14}C]Phe in final vol. 5 ml and put through a Sephadex G-25 column (2.5 × 10 cm), equilibrated with 50 mM potassium phosphate buffer (pH 7.2) containing 10% glycerol and 1 mM DTT. The protein fraction was concentrated in a Sartorius bag against 50 mM potassium phosphate buffer (pH 7.2) and 1 mM DTT to 1.5 ml. Subsequent incubation was carried out for 5 min at 37°C in total vol. 0.5 ml containing 0.2 ml concentrated protein fraction (40 μg, 975 cpm), 30 μg light enzyme, 5 mM D-phenylalanine, L-proline, L-valine, L-ornithine and L-leucine, and otherwise optimal conditions for gramicidin S synthesis.

3. Materials

L-[U-^{14}C]Phe (520 mCi/mmol) and D-[l-^{14}C]Phe (60 mCi/mmol) were purchased from Amersham. The purity was checked by two-dimensional thin-layer chromatography using the system in [5].

4. Results and discussion

It is seen from table 1 that the heavy enzyme catalyzes to a small but significant degree the ATP–$^{32}PP_i$ exchange reaction in the presence of L-Phe or D-Phe. Compared to L-Orn which is one of the normal substrates for this enzyme, the rate of the reaction is only about 1%. The demonstration of binding of L-[^{14}C]Phe and D-[^{14}C]Phe to the heavy enzyme in an acid stable linkage is shown in table 2. The degree of binding was about 10% L-Orn and 16% L-Orn. The

Table 1
ATP–$^{32}PP_i$ exchange reaction in the presence of L- and D-Phe catalyzed by the heavy enzyme

Amino acid	[^{32}P]ATP (cpm)
L-Phe	589
D-Phe	786
L-Orn	64416

The exchange reaction in the presence of L-Orn is included for comparison

Table 2
Thioester binding of D-[^{14}C]Phe and L-[^{14}C]Phe to the heavy enzyme

Amino acid	Thioester-bound radioact.	
	cpm	(%)
L-[^{14}C]Phe	1461	10
D-[^{14}C]Phe	2394	16
L-[^{14}C]Orn	15 235	100

The thioester binding of L-[^{14}C]Orn is included for comparison

labelled substance bound to the enzyme was liberated completely by treatment with performic acid and was shown by chromatography to be phenylalanine. This rules out the possibility that the enzyme converted the amino acid to some other substance which then became bound to the enzyme. Treatment of the heavy enzyme charged with [^{14}C]Phe with alkali also resulted in its complete removal from the enzyme.

Although polyacrylamide gel electrophoresis did not reveal the presence of any light enzyme in the heavy enzyme used (see section 2) the presence of small amounts of light enzyme in the heavy enzyme could not be ruled out completely. However, if small amounts of light enzyme was present in the heavy enzyme, gramicidin S should be formed when the heavy enzyme was incubated with all 5 constituent amino acids. It is seen from table 3 that this was not the case. Hence the heavy enzyme does activate and bind in thioester-linkage both D- and L-Phe.

To find out if the thiol site to which Phe becomes bound when incubated with the heavy enzyme partici-

Table 3
Attempted synthesis of gramicidin S using the heavy enzyme only

Incubation mixture		Gramicidin S incorporation
Enzyme	Amino [^{14}C]acid	(cpm)
Heavy enzyme	D-Phe	0
Heavy enzyme	L-Phe	0
Heavy + light enzyme	L-Phe	121.450

An incubation mixture containing the light enzyme in addition is included for comparison

pates in gramicidin S synthesis, the heavy enzyme was incubated with D-[^{14}C]Phe in order to charge it with this amino acid and the incubation mixture subsequently passed through a Sephadex G-25 column. D-Phe was used since it was assumed that the heavy enzyme could not racemize L-Phe. To the protein fraction Mg^{2+}, ATP, light enzyme, D-Phe, L-Pro, L-Val, L-Orn and L-Leu were added and incubated (see section 2). No synthesis of radioactive gramicidin S was found. Control experiment adding further labelled Phe showed that the heavy enzyme containing bound D-Phe had retained its ability to synthesize gramicidin S. Hence the thiol site on the heavy enzyme to which D-Phe is bound is not involved in gramicidin S synthesis. The significance of this thiol site on the enzyme is therefore not yet known.

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References

[1] Laland, S. G., Frøyshov, Ø., Gilhuus-Moe, C. C. and Zimmer, T. L. (1972) Nature 43–44.
[2] Christiansen, C., Aarstad, K., Zimmer, T. L. and Laland, S. G. (1977) FEBS Lett. 81, 121–124.
[3] Pass, L., Zimmer, T. L. and Laland, S. G. (1974) Eur. J. Biochem. 47, 607.
[4] Gevers, W., Kleinkauf, H. and Lipmann, F. (1968) Proc. Natl. Acad. Sci. USA 60, 269–276.
[5] Frøyshov, Ø., Zimmer, T. L. and Laland, S. G. (1970) FEBS Lett. 7, 68–71.